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Analysis of Puumala Hantavirus Genome in Patients With Nephropathia Epidemica and Rodent Carriers From the Sites of Infection

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Reverse transcription-polymerase chain reaction (RT-PCR) followed by sequence and phylogenetic analyses were used to study specimens from nine Finnish nephropathia epidemica (NE) patients admitted to hospital during the epidemic in winter 1996–1997. Blood samples from six patients were found to be positive for the partial M- and/or S-segment sequences of Puumala hantavirus (PUUV). Analyses of these sequences (nt 2168–2610 for the M segment, and nt 819–1082 for the S segment) revealed six distinct PUUV strains showing highest similarity to previously described PUUV strains from Finland: 90–95% for the S segment, and 90–99% for the M segment. Accordingly, on the phylogenetic trees calculated for both viral segments, all six human strains were placed within the Finnish genetic lineage of PUUV. Attempts were made to trace five RT-PCR-positive patients to local bank voles (*Clethrionomys glareolus*) infected with wild-type PUUV, and for two patients a comparative analysis of human- and rodent-originated viral sequences was undertaken. Whereas in the first case the differences between the sequences were substantial (5.7% for the S segment, and 10.8%, for the M segment), in the other case the M segment sequence recovered from the clinical specimen was 100% identical to three sequences recovered from rodent lungs, and the S sequences differed by one silent substitution only. This is the first finding of virtually identical PUUV sequences in an NE patient and a natural rodent host from the site of infection. ***J. Med. Virol. 59:397–405, 1999.***

KEY WORDS: bank vole and human hantavirus sequences; comparison

INTRODUCTION

Puumala virus (PUUV) belongs to the *Hantavirus* genus, family *Bunyaviridae.* The genome of PUUV consists of (L)arge, (M)edium, and (S)mall RNA segments of negative polarity encoding, respectively, the viral RNA polymerase, precursor for two surface glycoproteins (G1 and G2), and nucleocapsid protein (N) [for review, see Plyusnin et al., 1996; Schmaljohn, 1996]. PUUV is carried mainly by the bank vole (*Clethrionomys glareolus*), a rodent species widely spread throughout Europe [Macdonald and Barrett, 1993]. While the viral infection in this natural host is persistent [Yanagihara et al., 1985; Gavrilovskaya et al., 1990], in humans PUUV causes nephropathia epidemica (NE), a relatively mild form of hemorrhagic fever with renal syndrome (HFRS) [for review, see Kanerva et al., 1998]. As severe hemorrhages or hypotensive shock complicate NE rarely, its mortality (0.1–0.2%) is substantially lower than that of the hantavirus pulmonary syndrome (HPS) caused by Sin Nombre and related hantaviruses in the Americas [Schmaljohn and Hjelle, 1997] or of the more severe forms of HFRS, caused by Hantaan and Seoul hantaviruses in Asia [Lee, 1996], and Dobrava virus in the Balkans [Papa et al., 1998; Avsic-Zupanc et al., 1999]. Finland has the highest documented incidence globally of human hantavirus infections. The acute febrile illness of NE manifesting as renal failure requires about 1,000 hospital admissions annually and transient dialysis treatment in 5% of the patients [Mustonen et al., 1994]. Recovery is usually complete.

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Distribution of PUUV follows that of *C. glareolus:* the virus is endemic in Fennoscandia [Brummer-Korvenkontio et al., 1982; Niklasson and LeDuc, 1987; Lundkvist et al., 1998] and the European part of Russia [Chumakov et al., 1981], but also occurs in many other parts of Europe. Accordingly, there is a geographic clustering of PUUV genetic variants [Plyusnin et al., 1994, 1995b; Hörling et al., 1995, 1996; Bowen et al., 1995, 1997; Lundkvist et al., 1998] that seems to reflect the complicated history of migrations of bank voles through the post-glacial Europe. Recently, PUUV was found in *C. rufocanus* in Hokkaido, Japan [Kariwa et al., 1995], but neither human cases nor positive sera have been reported yet.

The occurrence of NE in humans depends strongly on the local pattern of population dynamics of the bank vole [Brummer-Korvenkontio et al., 1982; Niklasson et al., 1995], thus proving an epidemiological link between PUUV infection in the natural virus host and in humans. Humans are thought to acquire PUUV by inhalation of infectious aerosols of animal excreta during agricultural work, woodcutting, or recreational activities such as gardening and maintaining of summer cottages. For instance, farmers have both an increased seroprevalence for PUUV antibodies [Ahlm et al., 1998; Vapalahti et al., 1999] and risk of disease contraction [Vapalahti et al., 1999]. Further, an overall PUUV antibody seroprevalence of 11% was found in European mammalogists and the corresponding value for Finnish rodent specialists was 50% [Vapalahti et al., 1995b]. Thus, the frequency of human contacts with PUUV natural hosts in endemic areas seems to be the most important factor for this infection.

Genetic dissection of hantavirus host-specificity is at an early stage, and little is known about factors that determine or influence the virulence of PUUV to humans. As there is no reliable animal model for human PUUV infection yet, these questions can be addressed by comparing the genetic properties of the virus presented in clinical specimens to those of wild-type PUUV from the site of infection. Recently, an epidemiological linkage of PUUV genomic sequences from one human NE case and from *C. glareolus* trapped 1 year later was demonstrated at the putative site of infection [Plyusnin et al., 1997]. Analysis of PUUV S- and M-segment nucleotide sequences recovered from the clinical specimen and rodent lung tissue suggested a reassortant nature of the human strain. This result was in line with reports on several human HPS cases caused by reassortant strains of Sin Nombre hantavirus [Henderson et al., 1995; Li et al., 1995; Schmaljohn et al., 1995]. The aim of the present study was to compare nucleotide sequences of human PUUV strains with those from bank voles trapped during the same epidemiological season within putative areas of infection. The aim was to learn more about: (i) mutations in the PUUV genome related to the host-switch, and (ii) putative reassortant nature of human PUUV strains.

MATERIALS AND METHODS

Patients

Nine acutely ill NE patients, eight men and one woman aged 18–59 years (mean 37 years), all treated at Tampere University Hospital between November 1996 and January 1997, were included in the study. In most cases there was a clear possibility of a recent rodent contact while hunting, farming, or working in places were rodents were seen. All patients had a typical clinical course of NE, although the clinical severity differed. In each case the diagnosis was confirmed from an acute-phase serum by detecting the typical granular staining pattern in the immunofluorescent antibody test using PUUV-infected Vero E6 cells as antigen [Vapalahti et al., 1995a] and/or low-avidity IgG antibodies to PUUV [Hedman et al., 1991]. Transient blood leukocytosis was observed in three and thrombocytopenia in eight patients. All patients had proteinuria and microscopic hematuria. Reduced renal function as expressed by elevated serum creatinine level was present in six patients, of whom one needed dialysis therapy. All patients recovered.

Selected cases. Case A.M. (#3) was a 33-year-old man who came to the hospital on December 3, 1996. His main clinical symptoms were high fever, nausea, and transient visual disturbances. The patient was treated at hospital for 6-days during which time laboratory studies showed low haemoglobin level (112 g/L), elevated blood leukocyte count (11.5×10^9/L), decreased platelet count (107×10^9/L), and elevated C-reactive protein (CRP) level (86 g/L). Proteinuria and haematuria were found in urinalysis but serum creatinine was normal. The patient did not develop any severe complications.

Case M.K. (#9) was a 48-year-old man sent to the hospital on March 13, 1997 because of high fever, nausea, and headache. He did not have any severe complications of NE such as hypotension or haemorrhages. Laboratory studies showed low platelet count (84×10^9/L) and CRP reaction up to 72 mg/L. Urinalysis disclosed haematuria and proteinuria but serum creatinine remained normal during hospital care of 6 days.

Clinical specimens. Blood samples were taken by the patients' informed consent 6–12 days (mean 8 days) after the onset of the clinical symptoms of NE. Peripheral blood mononuclear cells (PBMC) were isolated in Leucoprep® tubes (Becton-Dickinson, Franklin Lakes, NJ) as recommended by the manufacturer. Briefly, 7–9 ml of blood anticoagulated either with lithium, heparin, or sodium citrate was centrifuged ($1{,}500 \times g$, 30 min), and the PBMC layer was collected and washed twice with RPMI-1640 medium. PBMC were pelleted and suspended in 1 ml of guanidinium thiocyanate solution for RNA extraction [Chomczynski and Sacchi, 1987]. RNA extracted from each specimen was then used in four to five independent amplification attempts (see below).

Rodents. Animal experimentation guidelines approved by the American Society of Mammalogists [Ad

hoc Committee on Acceptable Field Methods in Mammalogy, 1987] were followed in animal studies. Bank voles were trapped in two sessions, in March and April 1997. The previous year 1996 was an increase year in Häme province of Finland (where the trapping sites were) as well as in the whole central Finland; 1997 was a peak year. In other words, bank vole populations increased already until the fall of 1996 and remained high until winter 1997–1998. Thus, the trapping period was in the middle of a high-density phase.

The trapped voles were killed under anesthesia, and the lung tissues were removed and kept at −70°C. Small pieces (2–4 mm) of frozen tissues were used for immunoblotting and for RNA extraction. Rodent tissue specimens were analyzed for the presence of PUUV N antigen as described earlier [Plyusnin et al., 1995a]. RNA from the antigen-positive samples was extracted using guanidinium thiocyanate method [Chomczynski and Sacchi, 1987].

Trapping to investigate case #3 (A.M.). This person lives permanently in Haukijärvi, west of Tampere. He had been hunting raccoon dogs in Längelmäki and had been digging three different nest sites. The maximum distance between the nests was 4 km (the map was enclosed in the hunting notes). Trapping was done on April 23–25, 1997 within 150 m of each nest. Längelmäki is east of Tampere, and the distance between the supposed permanent residence and hunting place is about 80–90 km.

Trapping to investigate case #9 (M.K.). M.K. lives permanently in Tampere city but has a summer place in Aitoo village, Luopioinen. It is an old small cottage in bad shape that M. K. has been repairing actively, for example, crawling under the house for floor isolation, etc. A wood shack and also some other outdoor buildings have been repaired. The house is situated at forest edge. All the trappings were done on March 20–22, 1997 within 100 m from the house.

Reverse transcription-polymerase chain reaction (RT-PCR) amplification and sequencing. RT-PCR of the partial PUUV S and M segment sequences was performed as described earlier [Plyusnin et al., 1997]. For the partial M segment sequence, the outer primer pair A1 (AATCCATCTGAGGCTACACCGTCT, pos. 1793–1816) and C2 (CCAACTCCTGAACCCCATGC, pos. 3011–3030) was used. Nested PCR was performed with primers B1 (AACCCGGCAAATGAACAAGAA, pos. 2147–2167) and B2 (TTGTTGGAGAGGACCGAGGAAT, pos. 2611–2632). For the partial S segment sequence, the outer primer pair Sa31 (TCATTTGA(A/G)GA(G/T)ATCAATGGCAT, pos. 532–554) and PUU5 (CCCATTCCAACATAAACAGTAGG, pos. 1197–1219) was used. Nested PCR was performed with primers PUU2 (CCAGGCACACCAGCACAGGA, pos. 799–818) and Sa5 (GCTGTGCC[A/C]ACAGTCTTAGATGCC, pos. 1083–1106). As shown earlier [Plyusnin et al., 1997], this strategy allows efficient amplification of PUUV genome sequences suitable for genetic analyses. The PCR-amplicons were separated in agarose gels and purified with the QIAquick kit (QIAGEN GmbH). Direct sequencing was performed using ABI PRISM Dye Terminator sequencing kit (Perkin-Elmer Applied Biosystems Division PE/ABI, Foster City, CA) according to the manufacturer's instructions and the reactions were run on an ABI 373 A sequencer (PE/ABI).

Phylogenetic analysis was performed using PHYLIP [Felsenstein, 1993]. A total of 200 bootstrap replicates of the sequence data were obtained (Seqboot). Distance matrices were calculated using Kimura's 2 parameter model (Dnadist; ratio 2.0). The matrices were analyzed by the Fitch-Margoliash tree-fitting algorithm (Fitch). The bootstrap support percentages of particular branching points were calculated from these trees (Consense).

RESULTS

Genetic Analyses of PUUV Nucleotide Sequences Recovered From the Clinical Specimens

RT-PCR revealed three positive clinical specimens for both M- and S-segment nucleotide sequences of PUUV (Table I). In addition, one patient was positive for the PUUV S-sequence only and two for PUUV M-sequence only. Identical results were obtained in at least two independent RT-PCR experiments for each of the genome segments, and the reason why some of the samples show either S- or M- positivity remains unknown. Thus, altogether six of nine (67%) patients were found RT-PCR positive; notably, all three PCR-negative specimens were collected on days 9–12 after the clinical onset of the symptoms, which should be considered suboptimal.

Nucleotide sequences were determined successfully for all PCR amplicons. Paired sequence comparison (Table II) revealed that the six patients were infected with PUUV strains related to but distinct from all previously reported strains. Nucleotide sequences of the newly detected strains (which were designated as NE97-1, NE97-2, NE97-3, NE97-5, NE97-6, and NE97-9). showed genetic diversity up to 8% within the S segment, and up to 10.5% within the M segment. All these strains were most similar to previously described PUUV strains from Finland. Strains from Russia, Sweden, Norway, France, Germany, and Japan, which represent other genetic lineages of PUUV, showed higher diversity from the NE-97 strains, ranging from 14.4% to 19.8% for the S segment sequences, and from 14.4% to 19.4% for the M segment sequences.

Notably, the M sequence of the human strain NE6-97 was 99.1% identical (4 silent substitutions per 440 nucleotide detected) to that of the bank vole-originated strain PUU/Virrat/25Cg/95, which has been previously connected to another NE case treated during the epidemic of 1994–1995 [Plyusnin et al., 1997]. In the patient record there is a note that the patient had worked in Ylöjärvi village (about 10 km west of Tampere) repairing a storage house in which the workers had seen some bank voles. Also, the patient's family had at that time spent some weekends in Kuru village, which lies

TABLE I. Results of Genetic Analysis of Nine NE Cases (Epidemic 1996–1997)

Case[a]	Samples collected on the day	Analysis of clinical specimens: RT-PCR test for: S segment	Analysis of clinical specimens: RT-PCR test for: M segment	Analysis of bank voles at putative places of infection: Total number of trapped rodents	Analysis of bank voles at putative places of infection: N-antigen-positive	Analysis of bank voles at putative places of infection: RT-PCR test for: S segment	Analysis of bank voles at putative places of infection: RT-PCR test for: M segment
1	6	–	+	0	0	ND	ND
2	7	+	+	2	0	ND	ND
3	7	+	+	16[b]	2	+	+
4	10	–	–	No trapping			
5	7	+	–	No trapping			
6	9	–	+	No trapping			
7	12	–	–	32	21	+[c]	+[c]
8	9	–	–	No trapping			
9	9	+	+	8	4	+[d]	+[d]

ND, not done.
[a]The severity of the disease in the scale "mild–moderate–severe" was regarded as following: the cases 2, 3, 5, 6, 8, and 9 = mild; cases 1 and 4 = mild/moderate, and the case 7 (which required a dialysis therapy) = severe. Patients 1, 4, 5, 6, 7, and 8 had acute renal failure (elevated serum creatinine).
[b]Rodents were trapped at three spots.
[c]Five N-Ag positive samples were checked.
[d]Sequences were recovered from three specimens.

TABLE II. Sequence Identity Among PUU NE-97 Strains and Between PUU NE-97 Strains and Other PUU Strains

	Percent nucleotide sequence identity: NE-97 strains	Finland[a]	Sweden[b]	Norway[c]	Russia[d]	France[e]	Germany[f]	Japan[g]
S segment (nt 819–1082[h])	92.0–98.4	90.0–97.8	82.1–85.6	82.2–84.2	80.7–83.6	80.2–81.6	81.4–84.2	82.4–85.1
M segment (nt 2168–2610)	89.5–96.4	89.5–99.1	81.7–83.2	—	84.3–85.6	80.6–83.0	—	—

[a]Includes strains: Sotkamo, Puumala, Evo, Virrat, NE95-1, NE95-9, and NE95-10.
[b]Includes strains: Vindeln L20, Vindeln 4, Hundberget 36, Mellansel 49, Lungvik, Sundsvall 255, and Sollefteå 3.
[c]Includes strains: Eidsvoll 1124v, Eidsvoll 1138, and Vinstra 1109.
[d]Includes strains: Udmurtia 458, Udmurtia 894, Cg1820, and P360.
[e]Includes strain France 90-13.
[f]Includes strain Berkel.
[g]Includes strains: Kamiiso and Tobetsu.
[h]nt 853–1053 for NE97-2.

between Tampere and Virrat (about 40 km north of Tampere). This finding suggests that Virrat/25-like strain(s) are still in circulation within the Tampere-Virrat natural focus.

Confirming results of the sequence comparison, on phylogenetic trees calculated for the S- and M-segment sequences (Fig. 1), all newly detected human PUUV strains were located within the Puumala clade, and belonged to the Finnish genetic lineage. Notably, the strain NE97-6 formed a well-supported group with the earlier described strains Virrat/25 and NE95-9 (bootstrap probability 96%, Fig. 1B). Other genetic lineages of PUUV were represented by strains from France (90–13), Germany (Berkel), Russia (Cg1820, Udmurtia, Kazan), Japan (Tobetsu, Kamiiso), Norway (Eidsvol, Vinstra), and Sweden. Strains from Sweden belonged to two distinct lineages indicated as Sweden-NPB and Sweden-SPB. The first lineage (Vindeln, Hundberget, Hällnäss, Mellansel) includes variants originated north of the border line that separates areas populated by two distinct genetic types of bank voles [Hörling et al., 1996; Lundkvist et al., 1998]. The second lineage is represented by variants originated south of the border line (Sundsvall, Sollefteå, Gräsmark); these strains are clustered together with Norwegian strains.

Comparison of Human PUUV Sequences With Those of Bank Voles in Putative Sites of Infection

With the patients' informed consent, attempts were made to trace five human cases (##1, 2, 3, 7, and 9) to local bank voles infected with a wild-type PUUV. The trapping was successful in putative sites of infections for four patients (Table I). For case #2, only two bank voles were trapped in the area around the person's summer cottage, and neither of them was found positive for PUUV N-antigen (N-Ag). The trapping around the summer cottage of the patient #7 was most successful: 21 of 32 trapped bank voles were found N-Ag-positive. Unfortunately, this clinical sample remained RT-PCR negative in several repeated attempts.

Comparative analysis of human- and rodent-originated (wild-type) PUUV sequences was possible for two cases: #3 and #9. For the case #3, trapping resulted in altogether 16 rodents, of which two were N-Ag and RT-PCR positive. The S segment nucleotide sequences recovered from these two voles (No. 126 and 127) diverged only in the third position of the Val_{326}-codon (C or T). The M segment sequence was recovered

from one vole only (No. 126). Corresponding wild-type PUUV strains were designated as Puumala/Längelmäki/126Cg/97 and PUUV/Längelmäki/127Cg/97, or Läng126 and Läng127, for short.

Patient #9 had been repairing an old summerhouse (e.g., crawling under the house for floor isolation, etc.) and some other outdoor buildings in Aitoo village. Eight bank voles were trapped within 100 m of the house, and three were found positive for both PUUV N-Ag and M-/S- nucleotide sequences (one Ag+ sample turned out to be RT-PCR negative). As the sequences recovered from all three animals were identical, the corresponding wild PUUV strain was designated as PUUV/Aitoo/84Cg/97 (or Ait84, for short).

Comparison of human- and rodent-originated PUUV sequences for the NE case #3 revealed a diversity of 5.7% for the S segment, and 10.8% for the M segment. Accordingly, on the resulting phylogenetic trees (Fig. 1) the human strain NE97-3 was situated separately from bank vole strains Läng126 and Läng127. In contrast, for case #9 M-segment sequences recovered from human and rodent specimens were 100% identical, and the S-segment sequences differed at one position only: in the human sequence the third nucleotide in the Phe_{336}-codon was thymidine, while in the rodent sequences it was cytosine. In agreement with that, on phylogenetic trees (Fig. 1) the human strain NE97-9 and the bank vole strain Ait84 are located in the closest possible proximity, indicating a genetic link between the human NE case and the wild-type PUUV.

DISCUSSION

Frequency and Duration of PUUV Genome Detection in Clinical Specimens

Altogether six of nine patients were found RT-PCR positive. Although calculated from a rather small number of clinical samples, this frequency of PUUV genome detection (67%) is about 2-fold higher than that we achieved during the epidemic of 1994–1995 [Plyusnin et al., 1997]. With that sensitivity of the amplification test, the RT-PCR positivity seems to become less dependent on the severity of the disease, thus making a contribution of other factors (such as the time of specimen collection) more important. As pointed out already, the three RT-PCR-negative specimens were collected on days 9, 10, and 12 after the onset of symptoms (Table I). According to our previous experience, only half of the specimens collected on day 9, and none of those collected later, were PCR positive [Plyusnin et al., 1997]. In other words, our amplification test can be used for PUUV genome detection during the first week of the disease or, maybe, a few days longer. Even within this period, the level of viremia seems to be very close to the limit of the test sensitivity, because some of the samples repeatedly showed positivity for either S- or M- PUUV genome segments only. This could happen only if the number of targets, that is, S- or M- RNA templates which remain intact after the extraction, is low enough to make the test dependent on random sampling (RNA extracted from each specimen was consumed in four or five amplification attempts). To evaluate the real level of viremia during PUUV infection, a quantitative or semi-quantitative PCR test should be developed. Probably, the electrochemiluminescent quantitative PCR approach, which was used successfully to examine Sin Nombre hantavirus RNA transcription in Vero E6 cell culture [Hutchinson et al., 1996], would be useful here.

Even the current, more sensitive, version of our RT-PCR still cannot be recommended as a test for routine diagnostics of PUUV infection, possibly with the exception of complicated cases. This is because at present the RT-PCR is too tedious for routine purposes and superior serological tests exist.

Comparison of PUUV Sequences From NE Patients With Those From Infected Bank Voles

Nucleotide sequences of six human PUUV strains, NE97-1, NE97-2, NE97-3, NE97-5, NE97-6, and NE97-9, were most closely related to the previously reported Finnish PUUV strains originated either from bank voles (Sotkamo, Evo, Puumala, and Virrat) or patients (NE95-1, NE95-9, and NE95-10) (Table II). These strains share a common ancestor and form a well-supported genetic lineage distinct from other lineages within the PUUV genotype (Fig. 1). Such a distribution fits with the view on geographical clustering of genetic variants in hantaviruses, in general [Plyusnin et al., 1996; Morzunov et al., 1998], and PUUV, in particular [Plyusnin et al., 1994, 1995b; Hörling et al., 1996; Bowen et al., 1997; Lundkvist et al., 1998]. Interestingly, the human strain NE97-3 clustered together with the earlier described strain NE95-10 (bootstrap probability 88%, Fig. 1A); corresponding wild-type strain(s) for these two human variants still remains to be found.

One aim of the study was to compare nucleotide sequences of human and bank vole PUUV strains to learn about changes that might accompany the host-switch of the virus. Recently, it has been shown that a host-switch of wild-type PUUV forces selection for variants that had one or two mutations in the non-coding regions of the viral RNA S segment, but not M segment [Lundkvist et al., 1997]. For the case #9 described in the present study, the partial M-segment sequence recovered from human and rodent specimens was 100% identical, whereas the partial S-segment sequences differed only by one silent substitution, in the Phe_{336}-codon. Although bank voles are known as PUUV carriers since late 1970s [Brummer-Korvenkontio et al., 1980], this is the first finding of virtually identical viral sequences in a NE patient and natural rodent hosts. Unfortunately, the PUUV sequences recovered from clinical specimens are still short, and do not allow for more intensive comparative studies.

Earlier, based on the genetic analysis of two epidemiologically linked PUUV strains, one of human and another of bank vole origin, a reassortant origin for the human strain was suggested [Plyusnin et al., 1997]. For the case #9 from the present study, no such indications were obtained. Thus, a reassortant nature of a

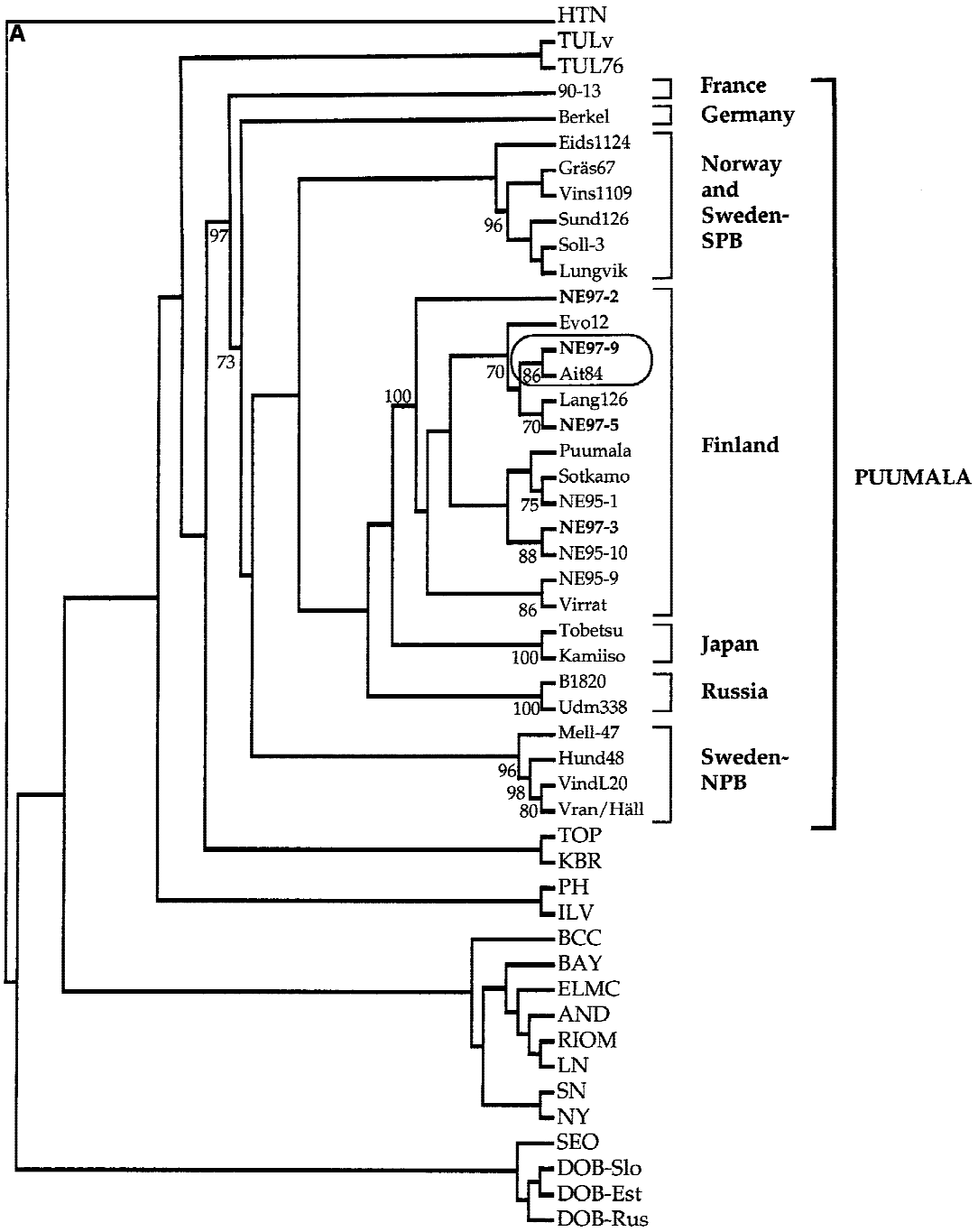

Fig. 1. **A:** Phylogenetic tree (consensus) of hantaviruses based on S-segment (nt 819–1082) nucleotide sequences. **B:** Phylogenetic tree (consensus) of hantaviruses based on the M-segment (nt 2168–2610) nucleotide sequences. Bootstrap probabilities of 70% and higher are shown for PUUV.

Abbreviations for Puumala virus strains: Sotkamo, Sotkamo/V-2969/81 (Gene Bank accession numbers X61035 and X61034 for S and M segments, respectively); Puumala, Puumala/1324Cg/79 (Z46942, for the S segment); Evo12, Evo/12Cg/93 (Z30702, for the S segment); Virrat, Virrat/25Cg/95 (Z69985 and Z70201); NE95-1, NE1/95 (Z69986, for the S segment); NE95-9, NE9/95 (Z69987 and Z69988); NE95-10, NE10/95 (Z69989 and Z69990); Udm338, Udmurtia/338Cg/92 (Z30708, for the S-segment); VindL20, Vindeln/L20Cg/83 (Z48586 and Z49214); B1820, Bashkiria/CG1820 (M32750 and M29979); Kazan, Kazan-wt (Z84205, for the M segment); Berkel, Berkel (L36943, for the S segment); 90-13, 90-13 (U22423 and U22418); Vran/Häll, "Vranica" (presumably Hällnäs B1) (U14137 and U14136); Eid1124, Eidsvoll/1124V (AJ223368 and AJ223367); Vins1109, Vinstra/1109Cg/86 (AJ223383, for the S segment); Hund48, Hundberget/48Cg/94 (AJ223372, for the S segment); Mell-47, Mellansel/47Cg/94 (AJ223374, for the S segment); Soll-3, Sollefteå/3Cg/94 (AJ223376, for the S segment); Lungvik, Lungvik/72Cg/95 (AJ223373, for the S segment); Sund126, Sundsvall/126Cg/95 (AJ223378, for the S segment); Gräs47, Gräsmark/47Cg/95 (AJ223370, for the S segment); Tobetsu, Tobetsu/60Cr/93 (AB010731, for the S segment); Kamiiso, Kamiiso/8Cr/95 (AB010730, for the S segment).

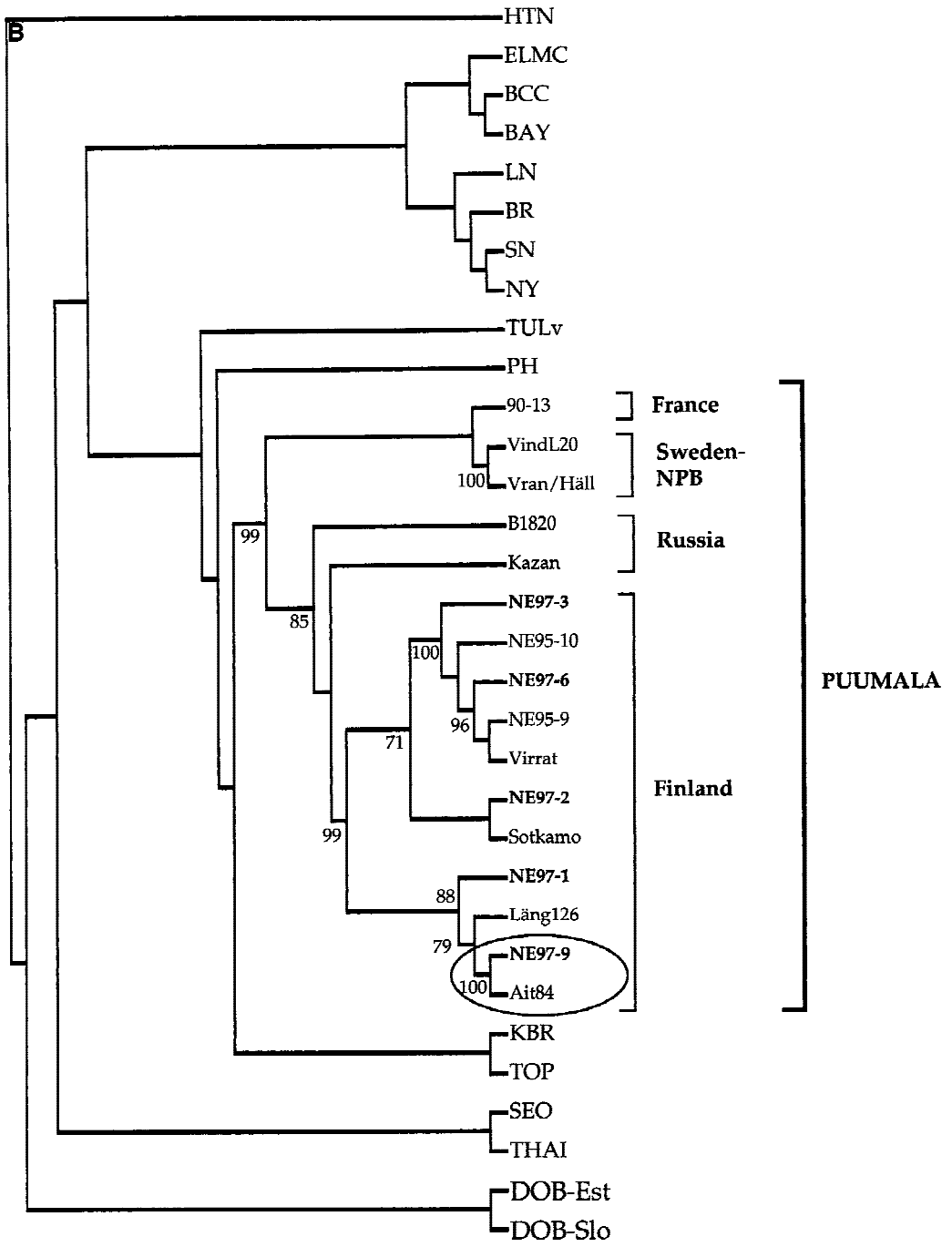

Fig. 1. Continued. Abbreviations for other hantaviruses: HTN, Hantaan virus, strain 76-118 (M14627 and M14627); SEO, Seoul virus, strain SR-11 (M34882 and M34881); DOB-Slo, Dobrava virus, strain Dobrava (L41916 and L33685); DOB-Est, Dobrava virus, strain Saaremaa/160V (AJ009773 and AJ009774); DOB-Rus, Dobrava virus, strain Kurkino/53Aa/98 (AJ131672, for the S segment); THAI, Thailand virus, strain 749 (L08756, for the M segment); TULv, Tula virus, strain Moravia/5302V/95 (Z69991 and Z69992); TUL76, Tula virus, strain Tula/Ma76/87 (Z30941, for the S segment); PH, Prospect Hill virus, strain PH-1 (Z49098 and Z55129); ILV, Isla Vista virus, strain MC-SB-1 (U31534, for the S segment); KBR, Khabarovsk virus, strain Mf43 (U35254 and U35255); TOP, Topografov virus, strain Topografov/Ls136V (AJ011646 and AJ011647); SN, Sin Nombre virus, strain H10 (L25784 and L25783); NY, New York virus, strain RI-1 (U09488 and U36801); BAY, Bayou virus, strain Louisiana (L36929 and L36930); ELMC, El Moro Canyon virus, strain RM-97 (U11427 and U26828); BCC, Black Creek Canal virus (L39949 and L39950); LN, Laguna Negra virus, strain 510B (AF005727 and AF005728); RIOM, Rio Mamore virus (U52136, for the S segment); AND, Andes virus, strain AH-1 (AF00466, for the S segment); BR, Blue River virus, strain Indiana (AF030551, for the M segment).

Other abbreviations: Sweden-SPB, Sweden, south of population border; Sweden-NPB, Sweden, north of population border [Hörling et al., 1996; Lundkvist et al., 1998].

PUUV strain does not seem to be obligatory for human infection. Similarly, not all HPS cases were associated with the Sin Nombre reassortants [Henderson et al., 1995]. The frequency and distribution of PUUV natural reassortants should be studied in more detail in the future.

Taken together, the results show that genetic analysis is an essential tool for "case-investigations" and mapping of natural microfoci of PUUV. Accumulation of experience in this field is important for evaluation of occupation- and habit-related risks of infection as well as for developing of proper strategy for vaccine development.

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NOTE ADDED IN PROOF

As it now appears that the Puumala virus strain 90-13 [Bowen et al., 1995] is actually Puumala virus strain CG 13891, a rodent isolate from Belgium [Van der Groen et al., 1987. Partial characterization of hantavirus isolated from a *Clethrionomys glareolus* captured in Belgium. Acta Virol. 31, 180–184], it was recommended that the strain designation "90-13" be discontinued.

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